Interaction of *Bifidobacterium animalis* Subspecies *lactis* (Bb12) and *Salmonella typhimurium* in Continuous-Flow Chemostatic Culture

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Abstract

A commercially available probiotic, *Bifidobacterium animalis* subspecies *lactis* (Bb12) was adapted to and maintained in a continuous-flow chemostat culture. We evaluated the growth characteristics and interactive effects of Bb12 and *Salmonella typhimurium* (St) when cultivated singly or together. When the continuous-flow culture of Bb12 was challenged with 10^4 to 10^7 CFU/ml of St, the St was eliminated within 24 h. This was replicated 3 times. Because the pH of the Bb12 was 4.5, it appeared that St elimination was due to the reduced pH. In a second study, St was grown in pure culture and the pH reduced to 4.5. Although still present, St concentrations dropped to unculturable levels within 28 h. In a third study, the pH of the Bb12 culture was maintained at pH 5.6 by means of a continuous drip of NaOH and challenged with St. Although at reduced concentrations (10^3 CFU/ml), the St remained in the chemostat until day 9 when the drip was discontinued. By day 14, the St was eliminated. It is apparent in these *in vitro* studies that Bb12 has antagonistic properties against St and it is possible that there could be some *in vivo* applications of Bb12 against St.

Introduction

Probiotics have been defined as live microorganisms that may beneficially affect the host (following ingestion) by improving the balance of the intestinal microflora. Some of the most commonly used probiotics contain lactic-acid-producing bacteria such as *Lactobacillus* and *Bifidobacterium* species (Lin, 2003). Among other effects, probiotics are purported to normalize the intestinal microflora, to reduce gut colonization by potential pathogens, to treat or prevent various types of diarrhea, to decrease the symptoms of irritable bowel syndrome and inflammatory bowel disease, and to modulate immune function (Lin, 2003).

Bifidobacterium are some of the earliest colonizers of the gastrointestinal (GI) tract of human infants and play an important role in the development of the permanent microflora. Many species of *Bifidobacterium* have been touted for health benefits, but *B. lactis* is probably the most commonly used. *B. lactis* conferred resistance to single or multiple oral challenges with virulent *Salmonella typhimurium* (St) in mice (Shu et al., 2000). The authors have viewed probiotics and mixtures of commensal bacteria as potential intervention strategies to increase immune function and as alternatives to antibiotics to control disease associated with enteropathogens such as *Salmonella* and enterotoxigenic *Escherichia coli* in swine (Harvey et al., 2002; Harvey et al., 2005). On the basis of the above-mentioned results of *B lactis* against St in mice (Shu et al., 2000), we hypothesized that a commercial probiotic of *Bifidobacterium animalis* subspecies *lactis* (Bb 12) might have antagonistic properties against *Salmonella* colonization in pigs. To test the potential of *in vivo* application, we decided that we must determine the *in vitro* effects of Bb12 against a swine isolate of St. The purpose of the present study was to evaluate the growth characteristics and the interactive effects of Bb12 and St when cultivated singly or together in an *in vitro* continuous-flow chemostatic model.

Materials and methods

B. animalis* subspecies *lactis. This strain of bacteria is commercially available as a probiotic (Bb12) and was obtained from Chr. Hansen, Inc. (Milwaukee, WI). A continuous-flow culture of Bb12 was adapted to Modified Reinforced Clostridia Media (MRCM), the culture established in a BioFlo 110 Fermentor (New Brunswick Scientific, Edison, NJ) using a 500 ml culture vessel with an exchange rate of 500 ml/day under anaerobic conditions. The MRCM consisted of pancreatic digest of casein (Casitone, 5.0 g/l), proteose peptone No. 3 (5.0 g/l), beef extract (10.0 g/l), yeast

extract (3.0 g/l), dextrose (5.0 g/l), NaCl (5.0 g/l), soluble starch (1.0 g/l), cysteine HCl (0.5 g/l), and sodium acetate (3.0 g/l).

Salmonella typhimurium (St). A primary porcine isolate of St, obtained from the National Veterinary Service Laboratories, Ames, IA, resistant to 20 µg/ml nalidixic acid (nal) and 25 µg/ml novobiocin (nov), was selected in our laboratory as the *Salmonella* challenge strain. For the continuous culture establishment of St control cultures, a 500 ml chemostat, filled with the MRCM was inoculated with St and grown under anaerobic conditions to achieve a final concentration of approximately 1 X 10^5 CFU/ml. The CFU of St were determined by serial dilution in phosphate buffered saline (PBS) and spread plating on BGA that contained 20 µg/ml nal and 25 µg/ml nov and incubated at 37° C for 24 h.

CFU determination of Bb12/St following challenge.

When Bb12 and St were grown in combination, a chemostat with an established steady-state culture of Bb12 would be inoculated (challenged) with an overnight culture of St. CFU of St were determined at 30 m, 4 h, 8 h, 24 h, and 48 h after St challenge. The procedures for CFU determination were described in the previous section. If BGA plates were negative for St growth, then 2.0 ml of the chemostat medium was added to tetrathionate enrichment broth and incubated at 37° C for 48 h. No growth after 48 h was considered a negative sample.

The CFU for Bb12 were determined by anaerobic serial dilution in PBS and spread plating on PRAS Bifido Selective Agar (Anaerobe Systems, Morgan Hill, CA) followed by incubation at 37° C for 24 h in a Bactron IV Anaerobic Chamber (Sheldon Manufacturing, Inc., Cornelius, OR). Samples for Bb12 CFU determination were collected at 30 m after St inoculation and every 24 h thereafter according to the serial dilution procedures outlined above.

Experimental design.

Study A. Challenge Bb12 with St

Replicate 1; challenge with 1 X 10^4 CFU/ml

Replicate 2; challenge with 1 X 10^5 CFU/ml, monitor pH at challenge and 24 h post-challenge

Replicate 3; challenge with 1 X 10^7 CFU/ml, monitor pH at 4, 8, and 24 h post-challenge

Study B. Effects of decreased pH on St growth.

To determine the effects of reduced pH on St growth, a chemostat that had reached a steady-state St concentration of 1 X 10^5 CFU/ml had concentrated HCl added every 2 h until a pH of 4.5 was reached. Serially diluted samples were streaked onto BGA, and if necessary, were enriched with tetrathionate broth. As mentioned above, a negative sample was one that was negative on BGA and had no growth after 48 h incubation in tetrathionate broth.

Study C. Effects of increased pH on St challenge of Bb12.

We hypothesized that increased pH in the chemostat could favor the colonization of St when grown in combination with Bb12. Following St challenge of an established culture of Bb12, we maintained a pH of 5.6 to 5.8 by the addition of sterile anaerobic NaOH (0.48 M) by continuous drip at a rate of approximately 82.0 ml/24 h (0.5%).

Results and Discussion

In study A, there was no growth of St on Brilliant Green Agar (BGA, Oxoid Ltd., Basingstoke, UK) at 24 h, 48 h, and 24 h post-challenge in replicates 1, 2, and 3, respectively, and were negative following enrichment. The pH of the Bb12 culture at challenge ranged from 4.42 to 4.50, before, during, and 24 h post-challenge in replicates 2 and 3.

Within 24 h in study B, St decreased from 10^6 CFU/ml at pH 5.6 to 10^3 CFU/ml at pH 4.5. By 28 h, the BGA plates were negative for St growth and continued to be negative though day 13. We continued to add hydrochloric acid (HCl) throughout 168 h (d 7), but at 192 h (d 8) we discontinued. The pH then slowly returned to 5.6 by day 13. Although BGA plates continued to be negative during this study, the tetrathionate-broth-enriched samples were consistently positive throughout day 13 when we terminated the study.

In study C, by 24 h post-challenge, St concentrations were 10^4 CFU/ml whereas Bb12 was at 10^8 CFU/ml. By day 9 when we turned off the continuous drip of sodium hydroxide (NaOH), the counts were 10^3 CFU/ml for St and 10^5 CFU/ml for Bb12. The pH went from 5.7 on day 9 to 4.7 on day 10 while the counts were 10^1 CFU/ml for St and 10^4 CFU/ml for Bb12. Beginning on day 11 and continuing through day 13, the sample for St was negative on BGA plates, but positive in

tetrathionate broth. On day 14, the tetrathionate-enriched samples were negative for St, the pH was at 4.4, and the Bb12 was 10^7 CFU/ml (See Figure). The study was terminated.

Figure 3

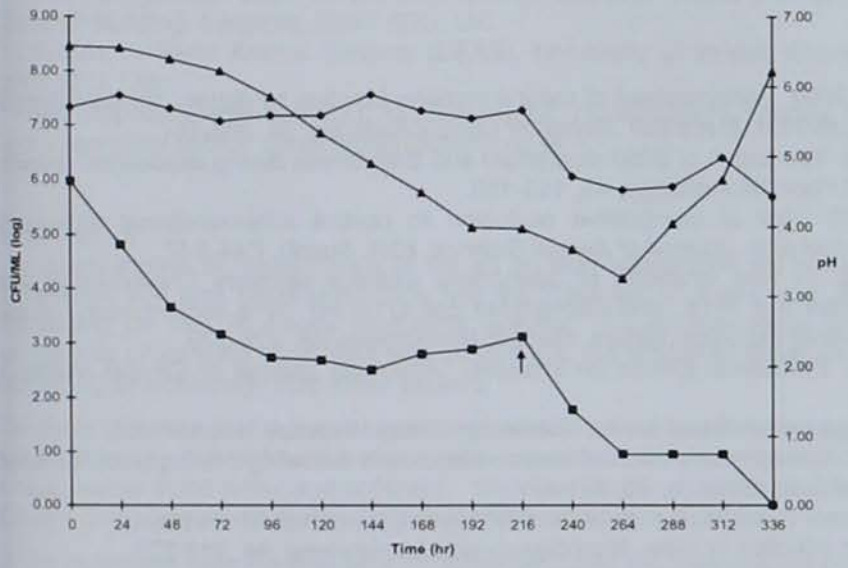

Figure. Study C: Interaction of Bb12 (▲) and St (■) when grown simultaneously in continuous -flow culture with pH (♦) at 5.6 to 5.8. pH was adjusted by continuous drip of NaOH. At 216 h (see arrow), the drip was discontinued.

In study A, Bb12 produced dramatic reductions of St in the chemostat. Because Bb12 produces lactic acid and the culture consistently maintained the pH at 4.42 to 4.50, we assumed that the elimination of St was due to the lowered pH. To test this hypothesis, we then conducted study B with St alone in a reduced pH environment. While the decrease in pH slowed the growth and reduced the CFU to a non-culturable level, pH alone did not sterilize the chemostat. Although pH appeared to have a great influence on St colonization, the results from this study showed that it is not the only factor in elimination. Hence, we designed study C in which increased pH should favor the growth of St and offset the acid-producing properties of Bb12. Study C suggested that Bb12 had some bacteriostatic properties against St because at 24 h post-challenge, St was 10^4 CFU/ml and by day 9 the CFU was 10^3. During that same time frame, Bb12 concentrations were also decreasing. Following the removal of the NaOH drip, Bb12 concentrations went up to 10^7 CFU/ml and St was eliminated from the chemostat.

While not conclusive, these results suggest that Bb12 may have some antagonistic properties against St. Our results are similar to another *in vitro* study (Bielecka et al., 1998) in which *B. animalis* was bactericidal to *S. enteritidis*. *B. lactis* has been shown to enhance resistance to oral challenge of mice with *S. typhimurium*, including a ten-fold survival rate in treated mice (Shu et al., 2000).

While the authors are not suggesting that *in vitro* data can be directly applied to *in vivo* conclusions, there are *in vivo* studies that show *B. lactis* (Bb12) added to infant formula had ameliorating effects on GI tract disease (Weizman, 2005), that consumption of *B. bifidum* (Bb12) increases leukocyte phagocytosis in human subjects (Schiffrin et al., 1995), that ingestion of *B. lactis* can enhance natural immunity in elderly human subjects (Arunachalam et al., 2000), and that probiotics such as *Bifidobacterium* have a major impact on the development and maintenance of immune function (Isolauri et al., 2001).

We do not know the mechanism of action for the antagonism of Bb12 on St, but it is known that early colonization of the GI tract by commensal bacteria can competitively exclude enteropathogens such as *Salmonella* and *Escherichia coli*. It has been said that commensals reduce pathogen colonization by competition for nutrients, competition for receptor sites,

stimulation of the immune system, and production of bacteriocidal products such as bacteriocins (Harvey et al., 2005).

We conclude that Bb12 can eliminate St in a continuous-flow chemostat culture and although it appears primarily due to reduced pH that inhibits St replication, our results suggest that other factors may play a role in St reduction. It is possible that there may be *in vivo* applications of Bb12 against St.

References

Arunachalam, K., et al., 2000. Enhancement of natural immune function by dietary consumption of *Bifidobacterium lactis* (HN019). *European Journal of Clinical Nutrition*, 54, 262-267.

Bielecka, M., et al., 1998. Interaction of *Bididobacterium* and *Salmonella* during associated growth. *International Journal of Food Microbiology*, 45, 151-155.

Harvey, R., et al., 2005. Use of competitive exclusion to control enterotoxigenic strains of *Escherichia coli* in weaned pigs. *Journal of Animal Science*, 83(E Suppl), E44-E47.

Harvey, R., et al., 2002. In vitro inhibition of *Salmonella enterica* serovars Choleraesuis and Typhimurium, *Escherichia coli* F-18, and *Escherichia coli* O157:H7 by a recombined porcine continuous-flow competitive exclusion culture. *Current Microbiology* 45, 226-229.

Isolauri, R., et al., 2001. Probiotics: Effects on immunity. *American Journal of Clinical Nutrition*, 73(Suppl), 444S-450S.

Lin, D.C. (2003). Probiotics as functional foods. Nutrition in Clinical Practice, 18, 497-506.

Schiffrin, E., et al., 1995. Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. *Journal of Dairy Science*, 78, 491-497.

Shu, Q., et al., 2000. Dietary *Bifidobacterium lactis* (HN019) enhances resistance to oral *Salmonella typhimurium* infection in mice. *Microbiology and Immunology*, 44, 213-222.

Weizman, Z., et al., 2005. Effect of a probiotic infant formula on infections in child care centers: Comparison of two probiotic agents. *Pediatrics*, 115, 5-9.